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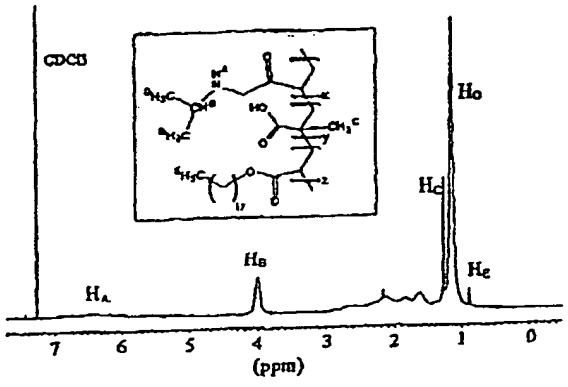
(54) Title: METHODS AND COMPOSITIONS FOR INCREASED POTENCY OF THERAPEUTIC AGENTS BASED ON PH-SENSITIVE POLYMERIC MICELLES

(57) Abstract: Novel polymeric micelles which are pH and/or temperature sensitive, and which are used to increase potency of therapeutic agents, including anti tumor drugs.




WO 01/87227 A2

# METHODS AND COMPOSITIONS FOR INCREASED POTENCY OF THERAPEUTIC AGENTS BASED ON PH-SENSITIVE POLYMERIC MICELLES

## FIELD OF THE INVENTION

The present invention relates to compositions comprising polymeric micelles which are useful for improving the potency of therapeutic agents, including, but not limited to, anticancer drugs.

## BACKGROUND OF THE INVENTION

A major obstacle associated with the use of chemotherapeutic agents is the lack of selectivity towards cancerous cells. This lack of selectivity has been linked to the toxic side effects of the use of such agents due to their delivery to both normal and abnormal cells. Lack of selectivity of drugs towards target cells is also a problem in the treatment of a variety of disorders in addition to cancer. Much research effort has focused on development of carriers for drugs that can selectively deliver the drug to target cells. For example, in order to improve the specific delivery of drugs with low therapeutic index, several drug carriers such as liposomes, microparticles, nano-associates and drug-polymer conjugates have been studied.

Of the targeting devices studied liposomes (phospholipid vesicles) have attracted considerable attention. Their targeting efficacy is, however, limited by quick scavenging by reticuloendothelial (RE) cells of the liver and spleen, instability in the plasma, limited capability at extravasation due to size, technical problems with their production and susceptibility to oxidation. Solutions to individual problems have been found, but a solution to more than one problem has never been combined in a single composition. For example, if recognition by RE cells is reduced and stability improved, it is very difficult to obtain liposomes having a diameter of less than 50 nm.

Alternative methods at improving the targeting potential have been investigated. Such methods have included

temperature sensitive liposomes, magnetic liposomes and pH sensitive liposomes. The rationale underlying the use of pH sensitive liposomes lies in the observation that the pH in tumoral tissue is lower than normal, and therefore the local concentration of drug could be increased since the liposomes would release their contents inside the tumor more readily than in other tissues. Another possibility would be that the liposomes would be destabilised after having penetrated the cells since the endosomes in which they end up following endocytosis is in itself acidic.

This approach has several problems associated with it. While the liposomes still need to avoid being picked up by RE cells, they also must be stable in plasma. In spite of this. pH-sensitive liposomes have been produced by combining polymorphic lipids such as unsaturated phosphatidylethanolamine with mildly acidic amphiphiles that act as stabilizers at neutral pH. These liposomes have been found to be relatively unstable or to rapidly lose their pH sensivity in plasma. Attempts to stabilize such liposomes by addition of PEGylated-lipids have resulted in a substantial loss of pH sensitivity. Further studies have centered on the use of fusogenic pH sensitive peptides. Here, the limitation stems from the immunogenic potential of such compounds.

Polymeric micelles were first proposed as drug carriers by Bader, H. et al in 1984. Angew. Makromol. Chem. 123/124 (1984) 457-485. Polymeric micelles have been the object of growing scientific attention, and have emerged as a potential carrier for drugs having poor water solubility because they can solubilize those drugs in their inner core and they offer attractive characteristics such as a generally small size (<100nm) and a propensity to evade scavenging by the reticuloendothelial system (RE).

Micelles are often compared to naturally occurring carriers such as viruses or lipoproteins. All three of these carriers demonstrate a similar core-shell structure that allows for their contents to be protected during



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transportation to the target cell, whether it is DNA for viruses or water-insoluble drugs for lipoproteins and micelles.

Lipoproteins were proposed as a vehicle for the targeting of antitumor compounds to cancer cells because tumors express an enhanced need for low density lipoproteins. The efficiency of lipoproteins as carriers has been questioned, however, mainly because drug-incorporated lipoproteins would also be recognized by healthy cells and because they would have to compete with natural lipoproteins for receptor sites on tumors. Conversely, viral carriers are mainly used for the delivery of genetic material and may have optimal use in applications that do not require repeated application of the delivery vehicle, since they are likely to elicit an immune response.

Polymeric micelles seem to be one of the most advantageous carriers for the delivery of water-insoluble drugs. Polymeric micelles are characterized by a core-shell structure. Pharmaceutical research on polymeric micelles has been mainly focused on copolymers having an A-B diblock structure with A, the hydrophilic shell moieties and B the hydrophobic core polymers, respectively. Multiblock copolymers such as poly(ethylene oxide)-poly(propylene oxide)- poly(ethylene oxide) (PEO-PPO-PEO) (A-B-A) can also self-organize into micelles, and have been described as potential drug carriers. Kabanov, A.V. et al, FEBS Lett. 258 (1989) 343-345. The hydrophobic core which generally consists of a biodegradable polymer such as a poly(β-benzyl-L-aspartate) (PBLA), poly (DL-lactic acid) (PDLLA) or poly (ε-caprolactone) (PCL), serves as a reservoir for an insoluble drug, protecting it from contact with the aqueous environment. The core may also consist of a water-soluble polymer, such as poly(aspartic acid) (P(Asp)), which is rendered hydrophobic by the chemical conjugation of a hydrophobic drug, or is formed through the association of two oppositely charged polyions (polyion complex micelles). Several studies describe the use of non- or poorly



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biodegradable polymers such as polystyrene (Pst) or poly(methyl methacrylate) (PMMA) as constituents of the inner core. See, e.g., Zhao, C.L. et al, Langmuir 6 (1990) 514-516; Zhang, L. et al, Science 268 (1995) 1728-1731 and Inoue, T. et al, J. Controlled Release 51 (1998) 221-229. In order to be considered as clinically relevant drug carriers, non-biodegradable polymers must be non-toxic and have a molecular weight sufficiently low to be excreted via the renal route. The hydrophobic inner core can also consist of a highly hydrophobic small chain such as an alkyl chain or a diacyllipid such as distearoyl phosphatidyl ethanolamine (DSPE). The hydrophobic chain can be either attached to one end of a polymer, or randomly distributed within the polymeric structure.

The shell is responsible for micelle stabilization and interactions with plasmatic proteins and cell membranes. It usually consists of chains of hydrophilic, non-biodegradable, biocompatible polymers such as PEO. The biodistribution of the carrier is mainly dictated by the nature of the hydrophilic shell. Other polymers such as poly(N-isopropylacrylamide) (PNIPA) and poly(alkylacrylic acid) impart temperature or pH sensitivity to the micelles, and could eventually be used to confer bioadhesive properties. Micelles presenting functional groups at their surface for conjugation with a targeting moiety are also known. See, e.g., Scholz, C. et al, Macromolecules 28 (1995) 7295-7297.

Polymeric micelles have been studied to determine whether potential exists for their use in the photodynamic therapy of cancer. Photodynamic therapy is a site-specific alternative to radio- and chemotherapy of solid malignancies which involves illumination of the lesions with visible light after systemic administration of a tumor-localizing photosensitizer. This results in the photooxidation of biomolecules, leading to microvascular stasis or direct tumor cell kill and, finally, to tumor necrosis and, eventually, tumor cure. See, e.g., Kessel, D., Drugs of Today 1996, 32, 385-396; Ochsner, M.. J. Photochem. Photobiol. B: Biology



ENSDOCID: <WO_____0187227A2_I_>

1997, 39, 1-18. A porphyrin photosensitizer, Photofrin™ (QLT Phototherapeutics, BC, Canada), presently approved for clinical use, has several disadvantages and this has led to the search for second generation photosensitizers offering greater chemical purity, better red light absorption and lower cutaneous photosensitivity. Among these dyes, the metallo-phthalocyanines have been studied extensively. See, *e.g.*, Spikes, J.E., Photochem. Photobiol. 1986, 43, 691-699; Rosenthal, I., Photochem. Photobiol. 1991, 53, 859-870. It has been shown that unsubstituted aluminum chloride phthalocyanine (AlClPc) is a better photosensitizer of tumor cells than its water-soluble sulfonated derivative, both *in vitro* and *in vivo*. See, *e.g.*, Ben-Hur, E.; Rosenthal, I., Photochem. Photobiol. 1986, 43, 615-619; Brasseur, N.; Ouellet, R.; La Madeleine, C.; van Lier, J.E. submitted 1999; Chan, W.S.; Brasseur, N.; Ouellet, ↑ .; van Lier, J.E., Eur. J. Cancer, 1997, Brasseur et al 1999 Cancer 80. 1533-1541 However, the photodynamic potentia ot been fully exploited due to its insolub al solvents. Conventional formulatio tants such as Cremophor EL (CRM) are pre ivo administration. Such surfactant formulations, however, have been shown to induce anaphylactic reactions in patients. See Dye, D.; Watkins, J. Br. Med. J., 1980, 280, 1353. Therefore, there remains a need for a method of delivery of effective photosensitizer agents which utilizes physiological solvents. The present invention provides novel polymeric micelles which are pH-sensitive and optionally may also be temperature-sensitive, and which may be used to formulate therapeutic agents, including photosensitizer agents in aqueous solutions.

## SUMMARY OF THE INVENTION

The present invention provides a micelle-forming composition, comprising:

a therapeutic agent; and



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a polymer which comprises at least one hydrophilic moiety, a pH-sensitive moiety and a hydrophobic moiety,

wherein said micelle comprises a hydrophobic core surrounded by a hydrophilic shell, and wherein said therapeutic agent is contained within said micelle.

The present invention further provides methods for loading the polymeric micelles with at least one suitable therapeutic agent.

The present invention also provides a polymeric micelle composition, comprising a therapeutic agent, wherein the therapeutic agent is protected from chemical interactions, such as hydrolysis, by being contained within the hydrophobic core of said micelle.

These and other features and advantages of the invention will be more readily understood by those of ordinary skill in the art from a reading of the following detailed description.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a colloidal composition consisting of polymeric micelles which may be used to deliver therapeutic agents which have poor water solubility. The polymeric micelles are characterized by a core shell structure, wherein a hydrophobic core is surrounded by a hydrophilic shell. The hydrophilic shell comprises a hydrophilic polymer or copolymer and a pH sensitive component.

The hydrophilic polymer or copolymer is preferably a compound which exhibits a lower critical solution temperature (LCST) between 30°C and 45°C. Preferred hydrophilic polymers or copolymers include, but are not limited to, poly(*N*-substituted acrylamides), poly(*N*-acryloyl pyrrolidine), poly(*N*-acryloyl piperidine), poly(*N*-acryl-L-amino acid amides), poly(ethyl oxazoline), methylcellulose, hydroxypropyl acrylate, hydroxyalkyl cellulose derivatives and poly(vinyl alcohol), poly (N-isopropylacrylamide), poly (N-vinyl-2-pyrrolidone), polyethyleneglycol derivatives, and



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combinations thereof. A particularly preferred hydrophilic polymer is poly (N-isopropylacrylamide) and its copolymers with N-vinyl-2-pyrrolidone.

The second component of the hydrophilic shell is an ionizable, pH-sensitive moiety. In preferred embodiments, the pH-sensitive moiety is an alkylacrylic acid, including, but not limited to methacrylic acid, ethylacrylic acid, propyl acrylic acid and butyl acrylic acid, or an amino acid such as glutamic acid.

The pH-sensitive moiety is utilized at a level of up to about 10 mol% of the polymeric micelle. When using methacrylic acid satisfactory results can be obtained using between about 2 mol% and about 5 mol%.

The hydrophobic moiety constitutes the core of the micelle. It may consist of a single alkyl chain, such as octadecyl acrylate or a double chain alkyl compound such as phosphatidylethanolamine or dioctadecylamine. The hydrophobic moiety may also be a water insoluble polymer such as a poly(lactic acid) or a poly(ε-caprolactone). The hydrophobic moiety is selected so as to have a mass of up to 10 kDa. It can be randomly distributed in the hydrophilic polymeric chain or attached to one end of the polymer. When the hydrophobic moiety is an alkyl chain, it should be present at a concentration varying between about 1mol% and about 5 mol%.

Polymeric micelles exhibiting pH-sensitive properties may also be formed by using common pH-sensitive polymers including, but not limited to copolymers from methacrylic acid, methacrylic acid esters and acrylic acid esters, polyvinyl acetate phthalate, hydroxypropyl methyl cellulose phthalate, cellulose acetate phthalate and cellulose acetate trimellitate. As stated above, in order to form micelles these polymers need to contain a hydrophobic moiety.

**Formation of Micelles**

Micelle formation occurs as a result of two forces. One is an attractive force that leads to the association of



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molecules, while the other is a repulsive force that prevents unlimited growth of the micelles to a distinct macroscopic phase. Amphiphilic copolymers self-associate when placed in a solvent that is selective for either the hydrophilic or hydrophobic polymer.

The micellization process of amphiphilic copolymers is similar to that for low molecular weight surfactants. At very low concentrations, the polymers exist only as single chains. As the concentration increases to reach a critical value called the critical association concentration ("CAC"), polymer chains start to associate to form micelles in such a way that the hydrophobic part of the copolymer will avoid contact with the aqueous media in which the polymer is diluted. At the CAC, an important quantity of solvent can be found inside the micellar core, and micelles are described as loose aggregates which exhibit larger size than micelles formed at higher concentrations. At those concentrations, the equilibrium will favor micelle formation, micelles will adopt their low energy state configuration and the remaining solvent will be gradually released from the hydrophobic core resulting in a decrease in micellar size. Amphiphilic copolymers usually exhibit a CAC which is much lower than that of low molecular weight surfactants. For example, the CAC of PEO-PBLA and PNIPA-PSt are between 0.0005-0.002%. Some amphiphilic copolymers, however, exhibit much higher CAC, reaching up to 0.01-10% in the case of poloxamers. Amphiphilic copolymers with high CAC may not be suitable as drug targeting devices since they are unstable in an aqueous environment and are easily dissociated upon dilution.

The micellization of amphiphilic copolymers can result in two different types of micelles depending on whether the hydrophobic chain is randomly bound to the hydrophilic polymer or grafted to one end of the hydrophilic chain. Micelles formed from randomly modified polymers are smaller than end-modified polymers. The micellar size is mainly determined by the hydrophobic forces which sequester the hydrophobic chains in the core, and by the excluded volume



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repulsion between the chains which limits their size. The difference in the balance of these two forces in random and end-modified copolymers may account for their different size. When terminal hydrophobic groups associate to form micelles, the water clusters immobilized around the hydrophobic segments are excluded from the core and no direct interaction exists between the core and the hydrophilic shell, which remains as mobile linear chains in the micellar structure. Randomly modified polymers, however, associate in such a manner that the hydrophobic and hydrophilic parts of the polymer are entangled together allowing possible contact between the core and the aqueous medium. This is an important issue, since exposed hydrophobic cores may result in secondary aggregation of polymeric micelles. Secondary aggregation has also been proposed as an hypothesis to explain the presence of large particles (>100nm) in micellar systems of PEO-P(Asp) bearing conjugated doxorubicin (DOX).

Determination of Critical Association Concentration (CAC)

Light scattering is widely used for the determination of the molecular weight and aggregation number of micelles. The onset of micellization can, however, be detected only if the CAC falls within the sensitivity of the scattering method. This is rarely the case for polymers in water. Gel permeation chromatography (GPC) under aqueous conditions can be employed since single chains and micellar fractions of copolymers exhibit different elution volumes. It is also possible to simultaneously determine by GPC the molecular weight of the micelles and their aggregation number. It is important that the integrity of polymeric micelles be maintained during their elution through the size exclusion column. Adsorption of the polymer on the column may also present a problem, especially at concentrations close to the CAC where micelles consist of large loose aggregates.

A preferred method to determine the CAC involves the use of fluorescent probes, among which pyrene is the most widely used. Pyrene is a condensed aromatic hydrocarbon that is



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highly hydrophobic and sensitive to the polarity of the surrounding environment. Below the CAC, pyrene is solubilized in water, a medium of high polarity. When micelles are formed, pyrene partitions preferentially toward the hydrophobic domain afforded by the micellar core, and thus experiences a non-polar environment. Consequently, numerous changes such as an increase in the fluorescence intensity, a change in the vibrational fine structure of the emission spectra, and a red shift of the (0,0) band in the excitation spectra are observed. The apparent CAC can be obtained from the plot of the fluorescence of pyrene, the $I_1/I_3$ ratio from emission spectra or the $I_{333}/I_{338}$ ratio from the excitation spectra versus concentration. A major change in the slope indicates the onset of micellization. The $I_1/I_3$ ratio is the intensity ratio between the first and third highest energy emission peaks and is measured at a constant excitation wavelength and variable emission wavelengths corresponding to $I_1$ and $I_3$. The CAC determined with fluorescence techniques needs to be carefully interpreted for two reasons. First, the concentration of pyrene should be kept extremely low ($10^{-7}$ M), so that a change in slope can be precisely detected as micellization occurs. Second, a gradual change in the fluorescence spectrum can sometimes be attributed to the presence of hydrophobic impurities or association of the probe with individual polymeric chains or premicellar aggregates. Changes in anisotropy of fluorescent probes have also been associated with the onset of micellization.

It has been demonstrated for several copolymers that the onset of micellization is mainly dependent upon the length of the hydrophobic polymer chains, and that the effect of the hydrophilic chain length on the CAC is less pronounced. The effect of the medium composition or loaded drug on the CAC may be difficult to predict. The CAC of methoxy PEO-PDLLA micelles remained the same in water, 0.9% saline solution and 5% dextrose solutions. (See X. Zhang et al, Int. J. Pharm. 132 (1996) 195-206.) This is not surprising considering the



BNSDOCID: <WO_____0187227A2_I_>

non-ionic nature of the polymer. We have surprisingly discovered, however, that the CAC of PNIPA copolymerized with octadecylacrylate and methacrylic acid was not significantly different in water and phosphate buffered saline (PBS), although these micelles have a negative zeta potential. Further, it was found that incorporation of 10% paclitaxel into MePEO-PDLLA micelles did not cause the CAC to change significantly. Thus a substantial amount of therapeutic agent may be loaded into the polymeric micelles of the invention without compromising their physical stability.

Polymeric micelles such as those of the compositions of the invention are characterized by their small size (10-100nm). Besides being needed for extravasation of the carrier materials, this small size permits the sterilization of the composition to be effected simply by filtration, and minimizes the risks of embolism in capillaries. This is not the situation encountered with larger drug carriers. Micellar size depends on several factors including copolymer molecular weight, relative proportion of hydrophilic and hydrophobic chains and aggregation number. The size of micelles prepared by dialysis can be affected by the organic solvent used to dissolve the polymer.

Determination of micelle size is particularly useful for the characterization of thermo-responsive micelles. Polymers used to prepare such micelles exhibit a lower critical solution temperature (LCST) which can be defined as the temperature at which the polymeric phase separates. Below the LCST the polymer is soluble, but it precipitates at temperatures above the LCST. The diameter of these micelles rapidly rises at temperatures above the LCST, due to hydrophobic interactions that result in the aggregation of the micelles. This effect of temperature on size has been shown to be reversible, since the micellar architecture was maintained after lowering the temperature below the LCST.

Micellar diameter and size polydispersity can be obtained directly in water or in an isotonic buffer by dynamic light scattering (DLS). DLS can also provide



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information on the sphericity of polymeric micelles. Using DLS it was shown that the addition of a low molecular weight surfactant such as sodium dodecyl sulfate (1% w/v) can destroy the polymeric micelle structure and bring about a complete shift of the mean diameter from approximately 50 to 3nm.

Micellar size can also be estimated by methods such as atomic force microscopy (AFM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM). These methods allow the characterization of the micelle shape and size dispersity. Ultracentrifugation velocity studies are sometimes performed to assess the polydispersity of polymeric micelles.

**Incorporation of Therapeutic Agents into Polymeric Micelles**

Loading of a therapeutic agent into the micelles can be realized according to techniques well known to one skilled in the art. For example, loading may be effected by dissolution of the compound in a solution containing preformed micelles, by the oil-in-water procedure or the dialysis method.

Therapeutic agents which may be used are any compounds, including the ones listed below, which can be entrapped, in a stable manner, in polymeric micelles and administered at a therapeutically effective dose. Preferably, the therapeutic agents used in accordance with the invention are hydrophobic in order to be efficiently loaded into the micelles. However it may be possible to form stable complexes between ionic micelles and oppositely charged hydrophilic compounds such as antisense oligonucleotides. Suitable drugs include antitumor compounds such as phthalocyanines (e.g. aluminum chloride phthalocyanine), anthracyclines (e.g. doxorubicin), poorly soluble antimetabolites (e.g. methotrexate, mitomycin, 5-fluorouracil) and alkylating agents (e.g. carmustine). Micelles may also contain taxanes such as paclitaxel.

Additional drugs which can be contained in micelles are conventional hydrophobic antibiotics and antifungal agents such as amphotericin B, poorly water soluble immunomodulators



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such as cyclosporin, poorly water soluble antiviral drugs such as HIV protease inhibitors and poorly water-soluble steroidal (e.g. dexamethasone), non-steroidal (e.g. indomethacin) anti-inflammatory drugs and genome fragments with or without a carrier.

Further, drugs can be incorporated into the polymeric micelle compositions of the invention by means of chemical conjugation or by physical entrapment through dialysis or emulsification techniques. The simple equilibration of the drug and micelles in water may not result in high levels of incorporated drug. Chemical conjugation implies the formation of a covalent bond, such as an amide bond, between specific groups on the drug and the hydrophobic polymer of the core. Such bonds are resistant to enzymatic cleavage mainly because of steric hindrance, and cannot be readily hydrolyzed unless spacer groups are introduced. Preferably, the therapeutic agent is incorporated by a physical entrapment procedure.

Hydrophilic compounds such as proteins may also be incorporated in the polymeric micelle compositions of the invention. The incorporation of such hydrophilic species may, however, require the chemical hydrophobization of the molecule. Polyionic compounds can be incorporated through the formation of polyionic complex micelles.

Physical entrapment of drugs is generally carried out by a dialysis or oil-in-water emulsion procedure. The dialysis method consists in bringing the drug and copolymer from a solvent in which they are both soluble, such as ethanol or *N,N*-dimethylformamide, to a solvent that is selective only for the hydrophilic part of the polymer, such as water. As the good solvent is replaced by the selective one, the hydrophobic portion of the polymer associates to form the micellar core incorporating the insoluble drug during the process. Complete removal of the organic solvent may be brought about by extending the dialysis over several days. In the oil-in-water emulsion method, a solution of the drug in a water-insoluble volatile solvent, such as chloroform, is



BNSDOCID: <WO_____0187227A2_I_>

added to an aqueous solution of the copolymer to form an oil-in-water emulsion. The micelle-drug conjugate is formed as the solvent evaporates. The main advantage of the dialysis procedure over the latter method is that the use of potentially toxic solvents such as chlorinated solvents can be avoided. Both dialysis and oil-in-water emulsion methods were compared for the incorporation of DOX in PEO-PBLA micelles. The emulsification method was more efficient since the DOX content of the micelles was estimated to be 12% (*w/w*) compared to 8% (*w/w*) for the dialysis technique.

The drug loading procedure may affect the distribution of a drug within the micelle. For example, Cao et al. (Macromolecules 24 (1991) 6300-6305), showed that pyrene incorporated in micelles as they were forming was not protected from the aqueous environment as well as pyrene incorporated after micelles were formed, although the first method yielded a drug loading three times higher than the second method.

Entrapment efficiency of the polymeric micelles of the invention depends on the initial amount of drug added. Exceeding the maximum loading capacity results in precipitation of the therapeutic agent, and consequently, lower yield. Further, efficiency of loading of the therapeutic agent depends on the aggregation number of copolymer. Micelles showing a higher aggregation number allow a greater amount of drug to be solubilized in their inner core.

**Examples of Therapeutic Agent-loaded polymeric micelles**

Examples of compounds loaded into polymeric micelles as well as the corresponding drug loading procedure are given in table 1. The polymeric micelle compositions of the invention are believed to be suitable for use as delivery systems for a wide range of therapeutic agents, including, but not limited to, anticancer drugs, plasmid DNA, antisense oligonucleotides or for the delivery of diagnostic agents to a specific organ in the body.



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Table 1

| Examples of drugs and tracers loaded into polymeric micelles | | | |
|---|---|---|---|
| Drug | Polymer | Incorporation Mode | Micelle size with drug (nm) |
| Amphotericin B | PEO-PBLA | P | 26 |
| Antisense oligonucleotide | PEO-P(Lys) | EA | 50 |
| Cisplatin | PEO-P(Asp) | C | 16 |
| Cyclophosphamide | PEO-P(Lys) | C | n.a. |
| Dequalinium | PEO-PE | P | 15 |
| Doxirubicin (DOX) | PEO-P(Asp) | C | 50 |
| DOX | PEO-P(Asp) | C | 14 - 131 |
| DOX | PEO-P(Asp) | C | 17 - 42 |
| DOX | PEO-PBLA | P | 30 |
| DOX | PEO-PDLLA | P | n.a. |
| DOX | PEO-PBLA | P | 37 |
| DOX | PEO-P(Asp) | P + C | n.a. |
| DOX | PNIPA-PBMA | P | n.a. |
| DOX | PAA-PMMA | P | n.a. |
| Gd-DTPA-PE $^{111}$In-DTPA-SA | PEO-PE | P | 20 |
| Haloperidol | PEO-PPO-PEO | P | n.a. |
| Haloperidol | PEO-PPO-PEO | P | 15 |
| Indomethacin | PEO-PBLA | P | 25 - 29 |
| Indomethacin | PEO-PCL | P | 145 - 165 |
| Indomethacin | PEO-PCL | P | 114 - 156 |
| Iodine derivative of benzoic acid | PEO-P(Lys) | C | 80 |
| KRN-5500 | PEO-PBLA | P | |
| | PEO-($C_{16}$,BLA) | | 71* |
| | PEO-P(Asp,BLA) | | |
| Paclitaxel | PEO-PDLLA | P | n.a. |
| Paclitaxel | LCC | P | <100 |
| Plasmid DNA | PEO-P(Lys) | EA | 140 - 150 |
| Soybean trypsin inhibitor | PEO-PE | P | 15 |



BNSDOCID: <WO_____0187227A2_I_>

| | | | |
|---|---|---|---|
| Testosterone | PEO-PDLLA | P | n.a. |
| Topoisomerase II inhibitor ellipticine | PEO-PE | P | n.a. |
| n.a.: not available, P: physical entrapment, C: chemical bonding, EA: electrostatic association<br>* After the sonication of PEO($C_{16}$, BLA) aggregates | | | |

Evidence of drug incorporation can be obtained by GPC or DLS since both methods detect changes in micellar size. The presence of drugs is usually associated with such an increase in the size of micelles. The location of a drug inside the micelle core may be demonstrated by quenching experiments. For instance, iodide (I) which is a water soluble quencher of DOX, does not affect the fluorescence of the micelle-incorporated drug but quenches the fluorescence of the free drug. Such experiments showed that DOX was retained in PEO-PBLA after freeze drying and reconstitution in water. In the case of DOX, the self-association of the drug in the micelle core also results in a decrease in the fluorescence intensity of the drug. Recently, the retention and slow release of amphotericin B from polymeric micelles was indirectly ascertained by measuring the decrease of its hemolytic activity after incorporation into PEO-PBLA micelles.

**Pharmaceutical applications**

The polymeric micelle compositions of the invention are suitable for use in a variety of pharmaceutical fields, such as oral delivery, sustained release and site-specific drug targeting. Preferably, the micelles of the invention are used as a transport for water-insoluble drugs. As illustrated by the example below, it has been surprisingly found that micelles of the invention, when used to transport an anticancer drug to malignant tumor cells, elicit a dramatic increase of the potency of the drug.

Without wishing to be bound by theory, it is believed that polymeric micelles may enter cells via the endocytic pathway. Release of drugs in the endosomes may prevent



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inactivation of the drug in the lysosomes. It is further believed that pH sensitive micelles may destabilise the endosomal membrane and provoke an intercellular distribution of the drug that is favorable to its pharmacological activity.

## EXAMPLES

The following examples are illustrative, and is not intended to limit the scope of the present invention.

### Example 1

**Materials.** N-isopropylacrylamide (NIPA), methacrylic acid (MAA), octadecyl acrylate (ODA) and 2-2'-azobisisobutyronitrile (AIBN) were purchased from Aldrich Chemical Co. (Milwaukee, WI). NIPA was dispersed in heptane, solubilized by acetone addition, and then allowed to recrystallize at 4°C prior to use. MAA was purified using an inhibitor remover disposable column for hydroquinone and monomethylether hydroquinone (Aldrich). AIBN was purified. AlClPc and zinc phthalocyanine (ZnPc) were received from Eastman Kodak (Rochester, NY) and Aldrich, respectively. Optical grade pyrene was purchased from Aldrich and used as received. All other chemicals were of analytical grade and used as received. 1,2-propanediol and CRM (polyethoxylated castor oil) were purchased from Sigma (St. Louis, MO).

**Synthesis and characterization of copolymer.** NIPA, MAA, ODA (at different molar ratios) and AIBN were dissolved in distilled 1,4-dioxane. The dioxane mass was 11 times the total monomer mass. The solution was degassed by bubbling $N_2$ for 15 min. Polymerization occurred as the solution was heated under stirring at 65°C for 5 hours. Polymers were recovered by precipitation in diethyl ether, resolubilized in tetrahydrofuran (THF), reprecipitated and washed extensively with diethyl ether. The polymers were then dissolved in water, filtered and freeze-dried. Their weight molecular weight was determined by gel permeation chromatography.



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Monodisperse polystyrene standards were used for calibration. Copolymer compositions were determined by $^1H$ NMR spectrometry and titration. $^1H$ NMR spectra were recorded on a Bruker AMX600 spectrometer in $CDCl_3$-deuterated chloroform solutions at 25°C with a relaxation time of 10 s. The MAA content of copolymer was assayed by titration.

**Determination of CAC.** Several polymeric solutions differing in polymer concentration but each containing $10^{-7}$M of pyrene were prepared and kept stirred overnight in the dark. CAC was determined in both water and phosphate-buffered saline (PBS). The polymeric solution in PBS was prepared each time by first dissolving the polymer in water. Fluorescence measurements were made after 5 min. under stirring at 20°C. The excitation wavelength was fixed at 336 nm. The values of $I_1/I_3$ emission peak ratios were plotted against polymer concentration. Below the CAC, pyrene is in a medium of high polarity and the value of the ratio is that of pyrene in water. When micelles are formed, pyrene partitions preferentially toward the hydrophobic domain afforded by the micellar core and experiences a non-polar environment which results in a decrease of the $I_1/I_3$ ratio.

**Phase transition pH.** A polymeric solution in ethanol was prepared and then dissolved in PBS or phosphate buffer 67 mM. The solution (25 mg/L) was stirred overnight at room temperature. Phase transition pH was determined by light scattering, using a 650 Perkin-Elmer fluorescence detector (Norwalk, CT). Emission and excitation wavelengths were 480 nm. Measurements were made after 6 min. under stirring at 37°C. The resulting intensities were plotted as a function of pH. Phase transition pH was determined from the intersection of a straight line going through the points on the rapidly-rising part of the plot and the abscissa axis. The $I_1/I_3$ ratio of pyrene ($10^{-7}$ M) was also assessed as a function of pH. The ratio was evaluated after 5 or 10 min. under stirring at 37°C in PBS.



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**Micelle size and zeta potential.** Size measurements were performed by photon correlation spectroscopy (PCS) using a Coulter N4 Plus (Hialeah, FL) in several media, namely, water, PBS, non-isotonic phosphate buffer 67 mM (pH 7.4), dextrose 5% (*w/v*) and isotonic saline solution (NaCl 0.9%, *w/v*). Micellar sizes were estimated at 62.6° or 90° scattering angle, before and after filtration through a 0.22-μm pore-size filter at 20°C and 37°C. Micellar size was measured for polymer containing 2 or 4 mol% ODA. Because of the lower solubility of the polymer containing 4 mol% ODA, the micelles were prepared by dissolving the polymer in an organic solvent. Polymeric solutions in ethanol, *N,N*-dimethylformamide (DMF) or THF were prepared, then dialyzed against water for 24 h using a Spectra/Por membrane MWCO 6-8000 (Spectrum Laboratories, Inc., Laguna Hills, CA). The stability of poly(NIPA-*co*-MAA-*co*-ODA) was also evaluated by measuring the variation of micellar size in water, phosphate buffer and PBS over a 2-month period. All samples were stored at 4°C. Electrophoretic mobility of the particles was determined by Laser-Doppler anemometry electrophoresis with a Coulter Delsa 440SX (Miami, FL) and transformed into zeta potential by applying the Smoluchowski equation. Duplicate measurements were performed in PBS and 10 mM NaCl, at 23°C.

**Incorporation of phthalocyanines.** The drug and the polymer were solubilized in DMF and dialyzed for 24 h, in the dark, against water using a Spectra/Por membrane MWCO 6000-8000 (Spectrum Laboratories Inc.). The solutions were filtered through a 0.22-μm pore-size filter and freeze-dried. Drug content was assayed by spectrophotometry at 670 nm using a Hewlett Packard 8452A diode array spectrophotometer (Boise, ID). Drug-loading (DL) and entrapment efficiency (EE) were determined using Eq 1 and Eq 2, respectively:

$$EE = \frac{\text{percent DL}}{\text{percent of initial content}} \times 100 \qquad \text{Eq 1.}$$

$$DL = \frac{\text{amount of drug in micelle}}{\text{amount of micelles}} \times 100 \qquad \text{Eq 2.}$$



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**Cellular photoinactivation.** Stock solutions of AlClPc-loaded PM (ALCLPc-PM) in dextrose 5% (*w/v*) and AlClPc in PBS containing 10% (*v/v*) CRM EL and 3% (*v/v*) 1,2-propanediol (AlClPc CRM) were prepared and filtered on a 0.22-$\mu$m filter. The solutions were then diluted to the desired concentration with Waymouth's medium (Gibco, Burlington, Canada) containing 1% fetal bovine serum (FBS) (ICN, Aurora, OH). EMT-6 mouse mammary tumor cells were maintained in Waymouth's medium supplemented with 15% FBS and 1% L-glutamin (Gibco). Cell survival was estimated by means of the colorimetric MTT (3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, 15 x $10^3$ EMT-6 cells per well were inoculated in 100 $\mu$L Waymouth's growth medium in 96 multi-well plates and incubated overnight at 37°C and 5% $CO_2$. The cells were rinsed twice with PBS and incubated with 100 $\mu$L of the drug (AlClPc CRM or AlClPc PM) at various concentrations in Waymouth's medium containing 1% FBS for 1 or 24 h at 37°C and 5% $CO_2$. After incubation, the cells were rinsed twice with PBS, refed with 100 $\mu$L Waymouth's medium and exposed to red light. The light source consisted of 2 500-W tungsten/halogen lamps (GTE Sylvania, Montreal, Canada) fitted with a circulating, refrigerated, aqueous Rhodamine filter. The fluence rate calculated over the absorbance peak of AlClPc (660-700 nm) was 10 mW $cm^{-2}$, and the plates were illuminated for 10 min for a total fluence of 6 $Jcm^{-2}$. The cells were incubated overnight at 37°C and 5% $CO_2$ before assessing cell viability. Fifty $\mu$L of a 5-fold diluted MTT stock solution (Aldrich, Milwaukee, WS; 5 mg/mL PBS) in Waymouth's medium was added to each well. After 3 h, 100 $\mu$L sodium dodecyl sulphate (Gibco) 10% in 0.01 N HCl was added in the wells. The plates were incubated overnight at 37°C, after which absorbance was read at 570 nm by means of a microplate reader (Molecular Devices, Thermo Max, Sunnyvale, CA). Average absorbance of the blank wells in which cells were omitted was subtracted from the readings of the other wells. Average absorbance of the control cells, which were incubated with dye-free Waymouth's 1% FBS, represents 100%



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cell survival. The extracellular drug dose required to inactivate 90% of cells ($LD_{90}$) was extrapolated from the survival curves. Eight-fold replicates were run peer drug and light dose, and each experiment was repeated at least 3 times.

**Results**

**Synthesis and characterization of copolymer.** Weight-average molecular weights were approximately 40,000 with a polydispersity index ($M_w/M_n$) of 2.7. The feed proportions of each comonomer were NIPA/MAA/ODA 93/5/2 or 91/5/4 mol%, and the corresponding random copolymers will be referred to as poly($NIPA_{93}$-co-$MAA_5$-co-$ODA_2$) and poly($NIPA_{91}$-co-$MAA_5$-co-$ODA_4$), respectively. MAA content, determined by titration, was similar to the feed composition. In Fig. 1, the corresponding signals of each monomer were retrieved in the $^1H$ NMR spectra. By NMR analysis, the proportion of ODA was obtained by comparing the signals at 4.03 ppm of the proton $H_B$ (NIPA) and 0.89 ppm of the proton $H_B$ (ODA). It was found that the ODA content of poly($NIPA_{93}$-co-$MAA_5$-co-$ODA_2$) and poly($NIPA_{91}$-co-$MAA_5$-co-$ODA_4$) was 3.1 and 6.2 mol%, respectively.

**Determination of CAC.** The apparent CAC was determined from the intersection of straight line segments drawn through points corresponding to the lowest polymer concentrations which lie on a nearly horizontal line with that going through the points on the rapidly-rising part of the plot. The value of the apparent CAC was found to be near 10 mg/L for polymers containing either 2 or 4 mol% ODA, whether the medium was water or PBS (Fig. 2). In the absence of ODA, the ratio $I_1/I_3$ remains fairly constant, confirming the absence of micelles (Fig. 2A). The CAC was not different in water and PBS, indicating that it is not affected by the presence of salts.



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**Phase transition pH** Poly (NIPA) is a thermo-responsive polymer which exhibits a lower critical solution temperature (LCST) of 32°C. At temperatures under the LCST, the polymer is soluble but precipitates if the temperature is raised above the LCST. By adding a small amount of hydrophilic titrable monomer (*e.g.* MAA), it is possible to increase the LCST of poly(NIPA) and make the polymer pH-sensitive. The polymer then becomes soluble at body temperature (37°C) but precipitates as the pH decreases. As shown in fig. 3, the polymer undergoes phase transition at a pH of 5.7-5.8 at 37°C. The presence of 2 or 4 mol% of ODA (Fig. 3A, 3B, 3D) does not seem to affect phase transition pH, since it remains similar in both cases and comparable to the phase transition pH of the copolymer without ODA (Fig. 3C).

A comparison of Fig. 3A and 3B, shows that phase transition pH is not significantly affected by the presence of NaCl 75 mM.

As the pH is lowered to reach values close to 5.5, the internal hydrophobic clusters of the micelles are destroyed, thereby creating an environment of higher polarity, which is reflected by the increased pyrene $I_1/I_3$ ratio (fig. 4).

**Micelle size and zeta potential.** Size measurements were carried out before and after filtration through a 0.22-$\mu$m pore-size filter. Before filtration, the poly($NIPA_{93}$-*co*-MAA-*co*-$ODA_2$) formulation showed the presence of 2 or more populations with a significant proportion of large aggregates (data not shown). After filtration, at 20°C and at a concentration of 5 g/L, micelle sizes were determined to be 13, 25, 31 and 35 nm in water, dextrose 5% (*w/v*), non-isotonic phosphate buffer pH 7.4 and PBS, respectively (Table 2).

At 37°C, micelle diameter in water remained small (19 nm), while it increased noticeably in PBS (over 400 nm) (Table 2). However, the increase in particle size was less pronounced when the polymeric solution in PBS was diluted (0.5 g/L), when NaCl 75 mM was removed or when the polymer



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was dissolved in dextrose 5% (w/v) (Table 3). The presence of electrolytes can change the hydration structure of the polymer and cause a reduction of hydrogen-bonded water, which is important for solubilization of the polymer. To evaluate the charge shielding of NaCl on the micelles zeta potentials were measured in NaCl 10 mM and PBS. The zeta potential of PM was higher in the buffer (pH 7.4). Electrolytes in PBS (75 mM NaCl) may compress the diffuse layer and therefore reduce the zeta potential of the polymeric micelles. The reduction in electrostatic repulsion may partly explain the micellar aggregation in PBS at 37°C. However, this salting out effect does not seem to induce the intermixing of NIPA chains with the internal core, as demonstrated by the low $I_1/I_3$ ratio of pyrene at pH 7.4 (Fig. 4), which indicates that pyrene is in a hydrophobic environment. Furthermore, the salting out effect differs from pH-induced precipitation of the polymer, in that no salt effect was observed at low polymer concentrations (Fig. 3A, 3B).



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Table 2

| Mean diameters of poly($NIPA_{93}$-co-$MAA_5$-co-$ODA_2$) micelles after filtration | | | | | | |
|---|---|---|---|---|---|---|
| Medium | Concentration (g/L) | Temperature (°C) | Angle (°) | Peak (%) | Peak mean (nm) | Peak S.D. (nm) |
| Phosphate buffer[A] | 5 | 20 | 62.6 | 100 | 30.9 | 10 |
| Phosphate buffer[A] | 5 | 37 | 62.6 | 100 | 89.6* | 57.6 |
| PBS | 0.5 | 37 | 90 | 100 | 165 | 34.6 |
| PBS | 5 | 20 | 62.6 | 100 | 35.4 | 20.9 |
| PBS | 5 | 37 | 62.6 | 100 | 404* | 367 |
| Water | 5 | 20 | 62.6 | 100 | 13.3 | 4.9 |
| Water | 5 | 37 | 62.6 | 72.0<br>28.0 | 19.7<br>222 | 9.7<br>10.6 |
| Dextrose | 5 | 20 | 62.6 | 85.7<br>14.3 | 25.5<br>1.0 | 7.5<br>0.3 |
| Dextrose | 5 | 37 | 62.6 | 100 | 58 4* | 31.6 |
| [A]pH 7.4, contains no NaCl<br>*Solutions in which visible aggregates are present | | | | | | |

Table 3

| Effect of storage on the mean diameter of poly($NIPA_{93}$-co-$MAA_5$-co-$ODA_2$) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Initial size | | | After 1 month | | | After 2 months | | |
| Medium: | Peak (%) | Peak mean (nm) | Peak S.D. (nm) | Peak (%) | Peak mean (nm) | Peak S.D. (nm) | Peak (%) | Peak mean (nm) | Peak S.D. (nm) |
| Phosphate buffer* | 100 | 30.9 | 10 | 73.4<br>26.6 | 29.1<br>230 | 8.2<br>73.4 | 100 | 915 | 206 |
| PBS | 100 | 35.4 | 20.9 | 79.5<br>20.5 | 27.4<br>1346 | 10.2<br>424 | 94.7<br>5.3 | 4643<br>104 | 135<br>8<br>392 |
| Water | 100 | 13.3 | 4.9 | 78.3<br>21.7 | 241<br>3.2 | 126.<br>3<br>1.5 | 95.6<br>4.4 | 3516<br>27.0 | 946<br>18.<br>7 |
| Polymer concentration: 5 g/L<br>* pH 7.4, contains no NaCl | | | | | | | | | |



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Because of their lower solubility in water, poly($NIPA_{91}$-*co*-$MAA_5$-*co*-$ODA_4$) micelles were prepared by the dialysis method. In water at 20°C, after filtration, the measured micelle diameters were 10 and 14 nm for micelles prepared with DMF and THF, respectively. However, when prepared in ethanol, micelles had a diameter of over 400 nm. For poly($NIPA_{93}$-co-$MAA_5$-co-$ODA_2$), the size of poly($NIPA_{91}$-co-$MAA_5$-co-$ODA_4$) micelles was dependent on the nature of the aqueous medium.

Over time, all micelle formulations showed signs of aggregation irrespective of the medium in which they were dissolved (Table 3). However, the increase in size was more pronounced in PBS than in water or non-isotonic phosphate buffer. Although the presence of aggregates could be detected, all solutions were still transparent, suggesting that the polymer remained in solution. Micellar aggregates could be dissociated after filtration through a 0.22-$\mu$m filter.

**Incorporation of phthalocyanines.** Incorporation of AlClPc into poly($NIPA_{93}$-co-$MAA_5$-co-$ODA_2$) micelles yielded an entrapment efficiency of 50-60% with DL reaching approximately 3% (*w/w*) (Fig. 5).

**Cellular photoinactivation.** No dark toxicity was observed with AlClPc formulated in pH sensitive polymeric micelles (PM) at the maximal concentrations tested, *i.e.* 10 $\mu$M AlClPc and 0.22 mg/mL PM. Upon light treatment, AlClPc PM induced greater photoactivity than AlClPc in Chremophor (CRM). The $LD_{90}$ with PM and CRM preparations was 0.1 $\mu$M and 2.85 $\mu$M after 1 h incubation, and 0.04 $\mu$M and 0.6 $\mu$M after 24 h incubation, respectively (Fig. 6). Cellular photoinactivation was found to be independent of drug loading since PM containing either 3% or 12% AlClPc induced similar phototoxicity. Furthermore, no difference in photoactivity was observed when the cells



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were pre-incubated for 5 min. with unloaded PM before the addition of AlClPc CRM, suggesting that enhanced photoefficiency of AlClPc PM as compared to AlClPc CRM does not result from increased membrane permeability in the presence of the polymer. Without wishing to be bound by theory, it is believed that the greater photoactivity of the PM preparation is related to the association of AlClPc with the polymer, and consequent cellular uptake and/or more efficient intracellular localization of AlClPc.

**Discussion**

It was surprisingly discovered that pH-sensitive PM could be prepared from hydrophobically-modified poly(NIPA) by adding hydrophilic titrable monomers, such as MAA. The polymer exhibits a low CAC as well as an acidic phase transition pH. PM made from the polymer containing 2 mol% ODA and 5 mol% MAA showed adequate water solubility and could be loaded with a substantial amount of the photoactive compound AlClPc. It was also surprisingly discovered that, *in vitro*, the PM formulation induced a greater degree of cellular photoinactivation than the CRM preparation. Without being bound by theory, it is believed that the pH-sensitive micelles of the invention can modify the intracellular distribution of the drug, thereby increasing its potency.

**Example 2**

**Preparation and Characterization of N-isopropylamide Copolymers for the Preparation of Ph-sensitive Polymeric Micelles**

**A. Preparation of the polymer DODA-polyNIPA-co-MAA**

Disuccinimidyl 4,4'-azobis(4-cyoanovalerate) (A-501) was prepared by mixing 4,4'-azobis(4-cyoanovaleric acid) (V-501) with 1.25 equivalents of *N*-hydroxysuccinimide in the presence of 1.5 equivalents of 1-ethyl-3-[3-(dimethylamino)-



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propyl]carbodiimide hydrochloride (EDC) in dry THF/$CH_3CN$ (1:1) at room temperature overnight. After solvent evaporation, A-501 was finely dispersed in a minimum of acetone. Cold water was slowly added to the dispersion and the solid was collected by filtration and washed with cold water.

The lipophilic radical initiator DODA-501 was prepared by the reaction of dioctadecylamine (recrystallized from acetone before use) with 0.8 equiv of A-501 in dry THF/$CHCl_3$ (2 :1). The product was purified by silica gel chromatography (mobile phase, hexane : ethyl acetate = 3 :1). The purified compound was finally precipitated in acetonitrile.

Before the polymerization, N-isopropylacrylamide (NIPA) was dispersed in heptane, solubilized by acetone addition, and then allowed to recrystallize at -20°C. Methacrylic acid (MAA) was purified using an inhibitor remover disposable column for hydroquinone and monomethylether hydroquinone (Aldrich).

DODA-poly(NIPA-*co*-MAA) was prepared by mixing NIPA, MAA and DODA-501 at a molar ratio of 96 :3 :1 (NIPA:1g, MAA:24$\mu$L, DODA-501:0.0592g) in 40 mL of anhydrous 1,4-dioxane. The solution was degassed by bubbling $N_2$ for 15 min and then heated at 69°C overnight. Polymer was recovered by several reprecipitations from diethylether, after solubilization in tetrahydrofuran (THF). After filtration, the polymer was dissolved in water and dialysed against water for 48h (MWCO 6000-8000, Spectrum Laboratories, Laguna Hills, CA). The suspension was filtered on 0.2 $\mu$m Nylon filter and then freeze-dried for 48h.

**Characterization of the copolymer**

The weight (Mw) and number-average (Mn) molecular weights of the polymer were determined by gel permeation chromatography in THF, using polystyrene standards for



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calibration. Mw and Mn were respectively 18000 and 8000 and the polydispersity index was 2.08.

Copolymer composition was determined by $^1H$ NMR spectrometry and titration The percentage of MAA was determined by acid-base titration of an ethanol polymer solution (1.6 mg/mL). The MAA content determined by titration was 4.6 mol% MAA.

By NMR analysis, the proportion of DODA was measured, by comparing the integration after deconvolution of the proton signal of NIPA (1.1 ppm) and the proton signal of DODA (0.9 ppm). It was found that the DODA content of $DODA_1$-poly($NIPA_{96}$-*co*-$MAA_3$) was 1.1 mol% DODA.

The phase transition pH of the polymer was determined by light scattering (480 nm) at 37°C using a Series 2 Aminco Bowman fluorimeter (Spectronics Instruments Inc., Rochester, NY). A polymeric solution in ethanol was prepared and then dissolved in PBS (25 mg/L). Measurements were taken after 5 min (in water, with stirring, at 37°C). The resulting intensities were plotted as a function of pH. Phase transition pH was determined from the intersection of a straight line going through points on the rapidly-rising part of the plot and the abscissa axis. The copolymer $DODA_1$-poly($NIPA_{96}$-*co*-$MAA_3$) starts to precipitate at pH 6.

The critical association concentration (CAC) of the copolymers was determined by a steady-state pyrene fluorescence method. Several polymeric solutions in water differing in polymer concentration but each containing $10^{-7}$ M pyrene were prepared and kept stirred overnight in the dark. Steady-state fluorescent spectra were measured ( at 390 nm) after 5 min under stirring at 20°C using a Series 2 Aminco Bowman fluorimeter (Spectronics Instruments Inc., Rochester, NY). The value of the CAC of the copolymer $DODA_1$-poly($NIPA_{96}$-*co*-$MAA_3$) was 33 mg/L.



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Size measurements were taken at 20°C by dynamic laser light scattering (DLS) using differential size distribution processor (SDP) analysis (Coulter N4 Plus, Hialeah, FL) in PBS (angle 90°). The micellar size was measured after filtration through a 0.22-$\mu$m pore-size filter. At a concentration of 7.5 mg/mL, micelle size of the copolymer $DODA_1$-poly($NIPA_{96}$-*co*-$MAA_3$) presented a bimodal distribution, with a small particle population of approximately 35 nm and larger aggregates around 100 nm.

**B. Preparation of the polymer DODA-poly(NIPA-*co*-MAA-*co*-VP)**

The lipophilic radical initiator DODA-501 was prepared as described in section A. The NIPA and MAA were purified with the method described in section A.

DODA-poly(NIPA-*co*-MAA-*co*-VP) was prepared by mixing NIPA, MAA, DODA-501 and vinyl pyrrolidone (VP) at a molar ratio of 88 :3 :1 :8 (NIPA:1g, MAA:26.3$\mu$L, DODA-501:0.0646g, VP:92.9$\mu$L) in 35 mL of anhydrous 1,4-dioxane. The solution was degassed by bubbling $N_2$ for 15 min and then heated at 69°C overnight. Polymer was recovered by several reprecipitations from diethylether, after solubilization in THF. After filtration, the polymer was dissolved in water and dialysed against water for 48h (MWCO 6000-8000, Spectrum Laboratories, Laguna Hills, CA). The suspension was filtered on 0.2 $\mu$m Nylon filter and then freeze-dried for 48h.

**Characterization of the copolymer**

The weight (Mw) and number-average (Mn) molecular weights of the polymer were determined by gel permeation chromatography in THF, using polystyrene standards for calibration. The Mw and Mn were 30000 and 14000 respectively, and the polydispersity index was 2.15.

Copolymer composition was determined by $^1H$ NMR spectrometry and titration. The percentage of MAA was determined by acid-base titration of an ethanol polymer



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solution (1.6 mg/mL). The MAA content determined by titration was 4.7 mol% MAA.

The proportion of DODA was measured by NMR analysis, by comparing the integration after deconvolution of the proton signal of NIPA (1.1 ppm) and the proton signal of DODA (0.9 ppm). It was found that the DODA content of $DODA_1$-poly($NIPA_{88}$-*co*-$MAA_3$-*co*-$VP_8$) was 1.4 mol% DODA. The proportion of VP was measured, by comparing the integration of the proton signal of NIPA (4.03 ppm) and the proton signal of VP (3.2 ppm). The VP proportion was found to be 4.9 mol%.

The phase transition pH of the polymer was determined by light scattering (480 nm) at 37°C as described in section A. The copolymer $DODA_1$-poly($NIPA_{88}$-*co*-$MAA_3$-*co*-$VP_8$) starts to precipitate at pH 5.6.

The critical association concentration (CAC) of the copolymers was determined by a steady-state pyrene fluorescence method described in section A. The value of the CAC of the copolymer $DODA_1$-poly($NIPA_{88}$-*co*-$MAA_3$-*co*-$VP_8$) was found to be 6 mg/L.

Size measurements were taken at 20°C as described in section A. At a concentration of 7.5 mg/mL, micelle size of the copolymer $DODA_1$-poly($NIPA_{88}$-*co*-$MAA_3$-*co*-$VP_8$) presented a bimodal distribution, with a small particle population of approximately 26 nm and larger aggregates of 100 to 350 nm.

**C. Preparation of the polymer poly(NIPA-*co*-ODA-*co*-MAA-*co*-VP)**

Poly(NIPA *co*-ODA-*co*-MAA-*co*-VP) was prepared by mixing NIPA, MAA, octadecylacrylate (ODA) (Aldrich, Milkauwee) and VP at a molar ratio of 87 :3 :2 :8 with 1,1'-azobis(cyclohexane carbonitrile) (Aldrich ACCN) used as an initiator (NIPA:1g, MAA:26.6μL, ODA:0.0659g, VP:93.9μL) in 35 mL of anhydrous 1,4-dioxane. Prior to polymerization, NIPA,



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MAA, were purified as described in section A. The solution was degassed by bubbling $N_2$ for 15 min and then heated at 69°C overnight. Polymer was recovered by several reprecipitations from diethylether, after solubilization in THF. After filtration, the polymer was dissolved in water and dialysed against water for 48h (MWCO 6000-8000, Spectrum Laboratories, Laguna Hills, CA). The suspension was filtered on a 0.2 μm Nylon filter and then freeze-dried for 48h.

**Characterization of the copolymer**

The weight- (Mw) and number-average (Mn) molecular weight of the polymer was determined by gel permeation chromatography in THF, using polystyrene standards for calibration. Mw and Mn were 26000 and 9000 respectively, and the polydispersity index was 2.8.

Copolymer composition was determined by $^1H$ NMR spectrometry and titration The percentage of MAA was determined by acid-base titration of an ethanol polymer solution (1.6 mg/mL). The MAA content determined by titration was 4.9 mol% MAA.

The proportion of ODA was measured using NMR analysis, by comparing the integration after deconvolution of the proton signal of NIPA (1.1 ppm) and the proton signal of ODA (0.9 ppm). It was found that the ODA content of poly($ODA_2$-*co*-$NIPA_{87}$-*co*-$MAA_3$-*co*-$VP_8$) was 2.8 mol% ODA. The proportion of VP was measured by comparing the integration of the proton signal of NIPA (4.03 ppm) and the proton signal of VP (3.2 ppm) and found to be 3.6 mol%.

The phase transition pH of the polymer was determined by light scattering (480 nm) at 37°C as described in section A. The copolymer poly($ODA_2$-*co*-$NIPA_{87}$-*co*-$MAA_3$-*co*-$VP_8$) starts to precipitate at pH 5.4.

The critical association concentration (CAC) of the copolymers was determined by a steady-state pyrene



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fluorescence method described in section A. The value of the CAC of the copolymer poly($ODA_2$-*co*-$NIPA_{87}$-*co*-$MAA_3$-*co*-$VP_8$) was found to be 10 mg/L.

Size measurements were taken at 20°C as described in section A. At a concentration of 7.5 mg/mL, micelle size of the copolymer poly($ODA_2$-*co*-$NIPA_{87}$-*co*-$MAA_3$-*co*-$VP_8$) presented a bimodal distribution, with a small particle population of approximately 30 nm and larger aggregates of 110 to 280 nm.



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THE CLAIMS

What is claimed is:

1. A micelle-forming composition, comprising:
a therapeutic agent; and
a polymer which comprises at least one hydrophilic moiety, a pH-sensitive moiety and a hydrophobic moiety,
wherein said micelle comprises a hydrophobic core surrounded by a hydrophilic shell, and
wherein said therapeutic agent is contained within said micelle.

2. The composition of claim 1, wherein the therapeutic agent is an anti tumor compound.

3. The composition of claim 2, wherein the anti tumor compound is selected from at least one phthalocyanine compound, anthracycline compound, antimetabolite, alkylating agent and taxane.

4. The composition of claim 3, wherein the phthalocyanine compound is aluminum chloride phthalocyanine.

5. The composition of claim 3, wherein the antimetabolite is selected from methotrexate, mitomycin and 5-fluorouracil.

6. The composition of claim 3, wherein the alkylating agent is carmustine.

7. The composition of claim 3, wherein the taxane is paclitaxel.

8. The composition of claim 1, wherein the therapeutic agent is selected from a hydrophobic antibiotic, a hydrophobic antifungal agent, an immunomodulator, an



BNSDOCID: <WO_____0187227A2_I_>

antiviral drug and a steroidal or non-steroidal anti-inflammatory drug.

9. The composition of claim 1, wherein the therapeutic agent comprises a genome fragment with or without a carrier.

10. The composition of claim 1, wherein the hydrophilic moiety is water soluble at a temperature of between about 35°C and about 40°C.

11. The composition of claim 10, wherein the hydrophilic moiety is water soluble at a temperature of about 37°C.

12. The composition of claim 10, wherein the hydrophilic moiety is selected from the group consisting of poly(*N*-substituted acrylamides), poly(*N*-acryloyl pyrrolidine), poly(*N*-acryloyl piperidine), poly(*N*-acryl-L-amino acid amides), poly(ethyl oxazoline), methylcellulose, hydroxypropyl acrylate, hydroxyalkyl cellulose derivatives and poly(vinyl alcohol) derivatives.

13. The composition of claim 1 wherein the hydrophilic moiety consists of poly (N-isopropylacrylamide) and one or more of the following compounds: N-vinyl-2-pyrrolidone and polyethyleneglycol.

14. The composition of claim 1, wherein the pH sensitive moiety is an alkylacrylic acid wherein the alkyl group contains from 1 to 24 carbon atoms, an amino acid or a pH sensitive polymeric material.

15. The composition of claim 14, wherein the pH sensitive moiety is selected from copolymers from methacrylic acid, methacrylic acid esters and acrylic acid esters, polyvinyl acetate phthalate, hydroxypropyl methyl cellulose



BNSDOCID: <WO_____0187227A2_I_>

phthalate, cellulose acetate phthalate and cellulose acetate trimellitate.

16. The composition of claim 14, wherein the amount of pH sensitive moiety is from about 0.5mol% to about 10mol%.

17. The composition of claim 14, wherein the pH sensitive moiety is methacrylic acid, and is utilized at from about 2mol% to about 5mol%.

18. The composition of claim 1, wherein the hydrophobic moiety has a mass of up to about 10kDA.

19. The composition of claim 18, wherein the hydrophobic moiety is an alkyl chain or a sterol.

20. The composition of claim 18, wherein the hydrophobic moiety is a water insoluble polymer.

21. The composition of claim 20, wherein the water insoluble polymer is a poly(lactic acid) or a poly (ε-caprolactone).

22. The composition of claim 18, wherein the hydrophobic moiety is utilized at a concentration of from about 0.1mol% to about 5mol%.

23. A pharmaceutical composition, wherein an effective amount of a therapeutic agent is entrapped within a polymeric micelle.

24. The composition of claim 23, wherein the therapeutic agent is entrapped within the polymeric micelles by means of dialysis or oil-in-water procedure.

25. The composition of claim 23, wherein the polymeric micelle is pH sensitive.



BNSDOCID. <WO_____0187227A2_I_>

26. A method for administering a therapeutic agent, comprising:

providing a micelle-forming composition of claim 1, and

administering the micelle-forming composition to a subject in need thereof.

27. A method for protecting a therapeutic agent comprising encapsulating said therapeutic agent within a micelle-forming composition.



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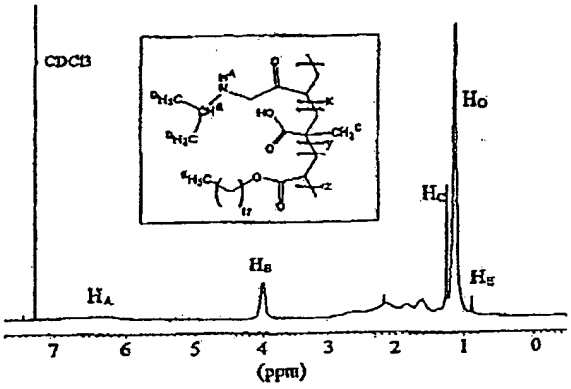


**Figure 1**

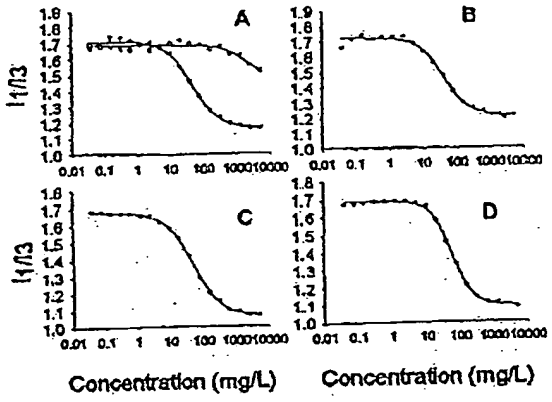


Figure 2

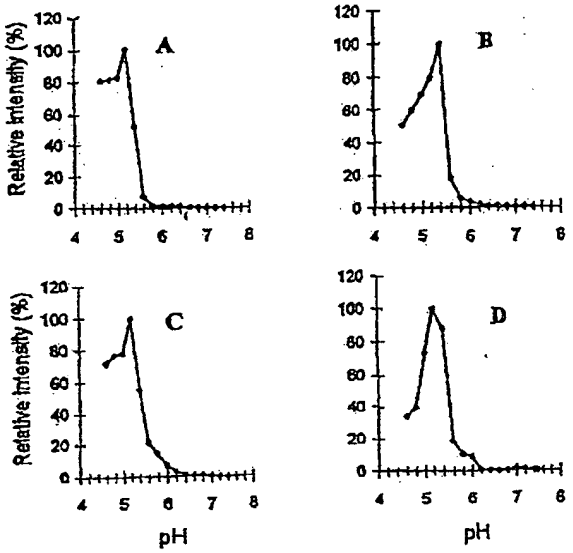
A
B
C
D
Relative Intensity (%)
Relative Intensity (%)
pH
pH
0
20
40
60
80
100
120
4
5
6
7
8

Figure 3

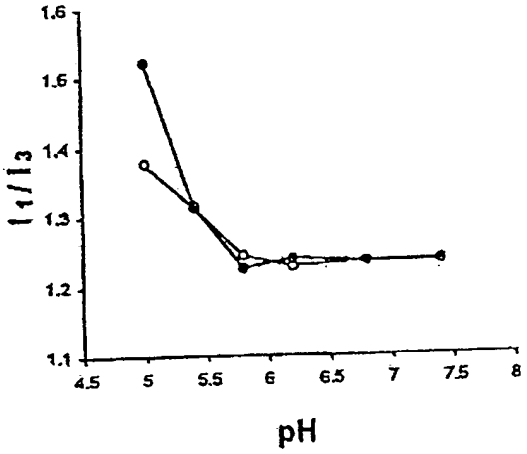


**Figure 4**

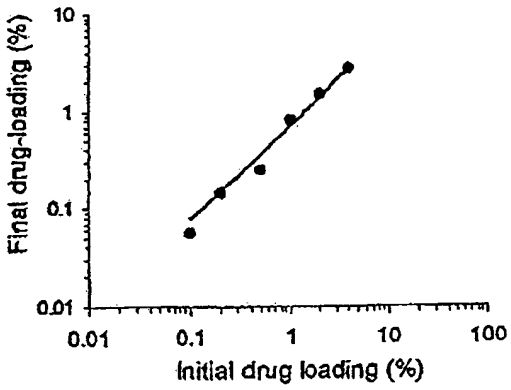
Final drug-loading (%)
10
1
0.1
0.01
0.01
0.1
1
10
100
Initial drug loading (%)

Figure 5

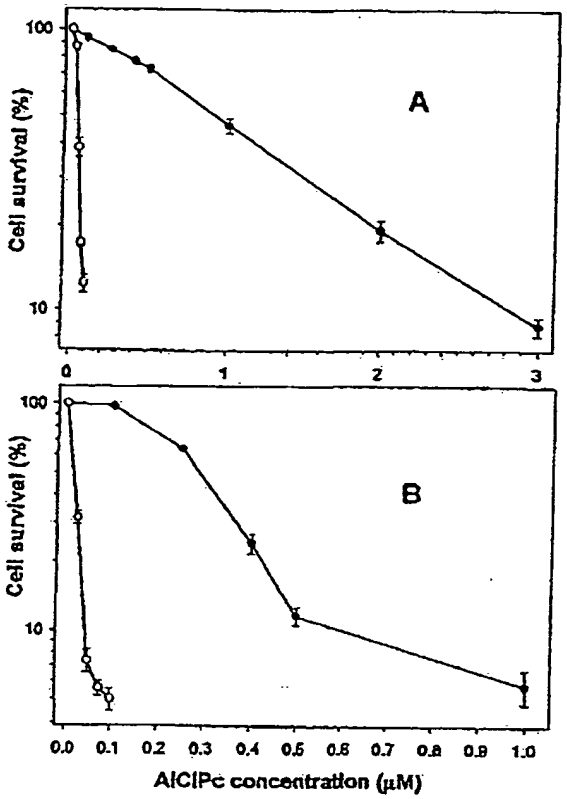
100
10
Cell survival (%)
A
0
1
2
3
100
10
Cell survival (%)
B
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
AlClPc concentration (μM)

Figure 6

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(74) Agent: AITKEN, David, W., Osler, Hoskin & Harcourt LLP, Suite 1500, 50 O'Connor Street, Ottawa, Ontario K1P 6LZ (CA).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

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(88) Date of publication of the international search report:
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*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 01/087227 A3

(54) Title: DRUG CONTAINING POLYMERIC MICELLES

(57) Abstract: Novel polymeric micelles which are pH and/or temperature sensitive, and which are used to increase potency of therapeutic agents, including anti tumor drugs.

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# INTERNATIONAL SEARCH REPORT

Inter ial Application No
PCT/IB 01/01456

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K9/107

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | J. TAILLEFER ET AL.: "Preparation and characterization of pH-responsive polymeric micelles for the delivery of photosensitizing anticancer drugs" JOURNAL OF PHARMACEUTICAL W SCIENCES, vol. 89, no. 1, January 2000 (2000-01), pages 52-62, XP002206433 Washington (US) | 1-4, 10-12, 14-20, 22-27 |
| Y | the whole document | 3,5-9, 21,24 |
| | --- | |
| | -/-- | |

[X] Further documents are listed in the continuation of box C.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 17 July 2002 | 01/08/2002 |

| Name and mailing address of the ISA | Authorized officer |
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| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Benz, K |

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| P,X | J-C LEROUX ET AL.: "N-isopropylacrylamide copolymers for the preparation of pH-sensitive liposomes and polymeric micelles" JOURNAL OF CONTROLLED RELEASE, vol. 72, no. 1-3, 14 May 2001 (2001-05-14), pages 71-84, XP004246437 Amsterdam (NL) page 79, paragraph 3.2. -page 81 | 1-4, 10-12, 14-19, 23-27 |
| X,O | & Sixth European Symposium on Controlled Drug Delivery; Noordwijk aan Zee, Netherlands; April 12-14, 2000 | |
| E | US 6 322 817 B1 (MAITRA ET AL.) 27 November 2001 (2001-11-27) column 6; example 3 | 1-3,7, 12,13, 23,25,26 |
| Y | M-C JONES ET AL.: "Polymeric micelles-a new generation of colloidal drug carriers" EUROPEAN JOURNAL OF PHARMACEUTICS AND BIOPHARMACEUTICS, vol. 48, no. 2, 1 September 1999 (1999-09-01), pages 101-111, XP004257101 Amsterdam (NL) the whole document | 3,7-9, 21,24 |
| Y | US 5 939 453 A (HELLER ET AL.) 17 August 1999 (1999-08-17) column 4, line 25 -column 5, line 8 column 11, line 27 -column 12, line 9 | 5-8 |



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# INTERNATIONAL SEARCH REPORT

ational application No.
PCT/IB 01/01456

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 26,27 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos..

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.



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# INTERNATIONAL SEARCH REPORT

ormation on patent family members

Inte nal Application No
PCT/IB 01/01456

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| US 6322817 B1 | 27-11-2001 | US 6365191 B1 | 02-04-2002 |
| US 5939453 A | 17-08-1999 | AU 4220499 A | 20-12-1999 |
| | | CN 1304423 T | 18-07-2001 |
| | | EP 1084170 A1 | 21-03-2001 |
| | | WO 9962983 A1 | 09-12-1999 |



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